Journal of Experimental Botany, Vol. 34, No. 144, pp. 927–935, July 1983

Inheritance of Naphthazarin Production and Pathogenicity to Pea in *Nectria haematococca*

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Received 30 September 1982

ABSTRACT

A naphthazarin-producing (fusarubin, novarubin, javanicin, norjavanicin), highly pathogenic strain of *Nectria haematococca* (sexual state of *Fusarium solani* var. *martii*) was crossed with a non-naphthazarin-producing, slightly pathogenic mutant strain. This cross and several backcrosses were studied by unordered tetrad analysis.

In all tetrads a 4:4 ratio of naphthazarin-producing ascospore strains to non-naphthazarin-producing ones resulted. By two-factor analysis for this character and the markers determining mating type, sexual type, colonial growth, and perithecial colour respectively, parental-ditype, non-parental-ditype, and tetratype tetrads resulted. This indicates that the loss of the capacity to produce naphthazarins is due to a mutation at a single locus, which probably blocks the biosynthesis of a polyketide precursor.

When the same tetrads were tested for pathogenicity, the strains could not be classified into a group with high pathogenicity and one with low pathogenicity, as they showed intermediate degrees of pathogenicity. It is therefore concluded that the degree of pathogenicity is polygenically inherited.

The difference in the inheritance of the capacity to produce naphthazarins and of pathogenicity, as well as the appearance of highly pathogenic though non-naphthazarin-producing segregants, indicates that the capacity to produce novarubin, fusarubin, javanicin, and norjavanicin is not a major factor in the determination of a high degree of pathogenicity.

Key words: Naphthazarin; Pathogenicity; Genetic.

INTRODUCTION

Fusarium spp. of the Martiella group cause root and stem rots in peas. *In vitro* these pathogens produce all or some of six toxic compounds: javanicin, fusarubin, novarubin, norjavanicin, marticin and isomarticin. The structure of these compounds is known and all have a naphthazarin structure (Kern and Naef-Roth, 1967). Naphthazarins, secondary metabolites of the polyketide type, are mainly produced by fungi and hardly ever by higher plants (Turner, 1971). They are all toxic to higher plants and micro-organisms but at different degrees. Marticin and isomarticin are predominantly toxic to higher plants, fusarubin, novarubin, javanicin and norjavanicin to bacterial growth and the latter two to fungal growth also. Marticin, isomarticin, and fusarubin can be extracted from diseased tissue (Kern and Naef-Roth, 1965).

During examination of the relationship between capacity to produce naphthazarins and pathogenicity, several strains of *Fusarium solani* var. *martii*, including artificial mutants, were compared with each other (Kern, 1976). Highly pathogenic strains were found to

produce greater concentrations of naphthazarin, especially marticins, than did weakly pathogenic strains. Mutants of a highly pathogenic strain of *Fusarium solani* var. *martii* which no longer produced naphthazarins were only weakly pathogenic.

This study is based on a cross between one of these mutants and a naphthazarin-producing (fusarubin, novarubin, norjavanicin, javanicin), highly pathogenic strain of *Nectria haematococca* which is the perfect state of *Fusarium solani* var. *martii*. Genetic analysis of the capacity to produce naphthazarins and of the degree of pathogenicity was facilitated by using the results of studies on the genetics of mating type, sexual reproduction, perithecial colour, growth types and fungicide tolerance in *Nectria haematococca*, reviewed by Snyder, Georgopoulos, Webster, and Smith (1975).

MATERIALS AND METHODS

Fungal strains

The following strains were used as parents:

Fusarium solani (Mart.) App. et Wr. var. *martii* (App. et Wr.) Wr. (synonym: *F. martii* var. *pisi*) ETH-strain No. M 808–1, highly pathogenic and highly toxigenic (Kern and Naef-Roth, 1965) and ETH-strain No. M 808–15, weakly pathogenic and weakly toxigenic mutant of M 808–1 (Dorn, 1974) obtained after treatment of M 808–1 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Nectria haematococca Berk. et Br. (mating population VI; Van Etten, 1978), strain No. 6–36, mating type + and strain No. 6–94, mating type −. These two strains are hermaphrodite, have sporodochial type and white perithecia. This species has unifactorial incompatibility with two mating types and two sex types, unisexual male and hermaphrodite. These strains were kindly provided by Dr. H. D. Van Etten.

Mating procedure and isolation of ascospores

Cultures were grown on V8-vegetable juice medium in test tubes (15 cm × 1·5 cm) at 24 °C under fluorescent lighting (Philips TLF 20W/29) for 7 d. Crosses were made by pouring 10 ml of a conidial suspension of one isolate onto the surface of another. After 15 min the spore suspension was decanted and the fertilized cultures were incubated as above (Van Etten, 1978). After 2–3 weeks, perithecia developed in fertile crosses. For ascospore isolation single perithecia were transferred into a drop of water to wash their surface clean of conidia. The perithecia were then pressed with fine forceps to expel the asci. Under a dissecting microscope (magnification 80×), the eight ascospores of a single ascus were isolated and singly transferred to a marked spot on a Petri dish containing malt agar medium. After 12 h small agar blocks, each with one germinated ascospore, were cut from the agar and transferred into individual tubes of malt agar medium.

Determination of genetic markers

The eight strains obtained from each ascus were tested for mating type, sexual type, growth type and perithecial colour (Table 1). *Mating type* and *sexual type* were determined by using each isolate as recipient or donor in crosses with strains of both mating types. The *growth type* of each isolate, whether sporodochial or mycelial, was examined after 10–15 d of growth. The *perithecial colour* depends only on the genotype of the recipient (female) strain. The allele concerned can only be determined in female isolates crossed with appropriate partner strains. The allele for perithecial colour of male strains must be evaluated by analysing the female isolates of the progeny of a cross between the male strain and a compatible partner strain. For this purpose these female F_1-isolates had to be fertilized with partner strains of opposite mating type.

Naphthazarin production and identification of the derivatives

An inoculum of each ascospore isolate (3 plugs of a 6-d old malt agar culture) was transferred into a 100 ml Erlenmeyer flask containing 20 ml of Raulin medium (Dorn, 1974). These cultures were incubated at 27 °C in the dark. After 5 d the cultures were filtered through paper and teflon (0·6 μm) to eliminate the spores. The culture filtrates were lyophilized. Each of the dry filtrates was redissolved in 5·0 ml of a solution of 100 parts of methanol and 5 parts of N HCl. Samples from these solutions of 50 μl each were layered on a silica-gel plate (Kieselgel 60, Merck No. 5553) in a line 12 mm long. The chromatograms were developed in a liquid phase of freshly prepared CCl_4:$HCCl_3$:HAc (20:10:3) (Roos, 1976). Quantitative measurements of the bands of the naphthazarin derivatives were made on a

TABLE 1. *Genetic markers of* Nectria haematococca

Symbol	Name	Alleles	References
a	mating type	a^+ = + polar a = − polar	Hansen and Snyder, 1943.
c	sexual type	c^+ = hermaphrodite c = unisexual male	Georgopoulos, 1963.
spo[a]	growth type	spo^+ = sporodochial spo = mycelial	Hansen and Snyder, 1943.
w	perithecial colour	w^+ = red perithecia w = white perithecia	Snyder, 1940.
nap	naphthazarin production	nap^+ = capacity to produce naphthazarins nap = loss of capacity to produce naphthazarins	

[a] The original symbols C for sporodochial and M for mycelial growth type (Hansen and Snyder, 1943) were changed into spo^+ (C) and spo (M), to avoid confusion with similar symbols.

spectrophotometer (Zeiss PMQII) at a wavelength of 500 nm, the wavelength at which these compounds have a maximal absorption of light (Pfiffner, 1963).

Degree of pathogenicity

The degree of pathogenicity was measured by an assay based on the size of lesions in pea stems (modified after Van Etten, 1980). Plastic pots were filled with sterile sand (2·0–2·5 mm diameter) and 20 ml of KNOP-solution (Dorn, 1974). Pea seeds (Alaska) were surface-sterilized with 0·1% $HgCl_2$-solution for 4 min and rinsed thoroughly with sterile water. Five seeds were placed on the sand in each pot and covered with additional sterile sand. Seedlings were grown for 7 d in a moist chamber under the following conditions: 16 h light (Philips TLF 55) at 25 °C; 8 h darkness at 18 °C; 70% r.h. The plants were inoculated by pressing the mycelial surface of an inoculum disc (3·0 mm diameter) onto a wound (0·5 mm deep and 5·0 mm long) made in the stem with a needle 2·0 cm above the sand surface. The inoculum discs were prepared by inoculating the surface of water agar with a plug of an older culture on malt agar, incubating the cultures for 5 d at 24 °C and cutting the discs at the edge of the growing mycelium. The inoculated plants were covered with a plastic bag, returned to the illuminated moist chamber and after 12 d the length of the lesions was measured. Ten seedlings were used for each isolate in each experiment.

Unordered tetrad analysis

As the ascospore positions in the asci of *Nectria haematococca* do not provide reliable information concerning the sequence of meiotic events (Georgopoulos, 1963), data were obtained by the analysis of unordered tetrads. When two pairs of alleles are segregating, three tetrad types can be recognized; parental ditype, non-parental ditype, and tetratype. The proportions of the three types of tetrads provide information about the linkage relationship of loci. Evidence for independent segregation of two loci is given by equal numbers of parental and non-parental ditype tetrads. A X^2-test was used to test equality. If the number of parental ditype tetrads exceeds that of non-parental ditype tetrads, then the two loci are likely to be situated in the same linkage group.

The tetratype tetrad frequencies of three unlinked loci allow the calculation of the post-reduction frequency (*PRF*) by using the Whitehouse formula. The *PRF* is a measure of the distance between a locus and its centromere (Burnett, 1975, p. 60). It is used in unordered tetrads instead of the second division frequency. The distance between two linked loci can then be calculated by subtracting the two respecting *PRF* values, if both loci are of the same arm of the chromosome; by the addition of the *PRF* values, if they are on opposite arms of the chromosome (Esser and Kühnen, 1965).

RESULTS

Genetic analysis

Genetic data presented in this paper were derived by two-factor analysis of unordered tetrads obtained in the 6 crosses mentioned in Table 2. All markers known previously (compatibility,

TABLE 2. *Crosses for two-factor analysis*

Crosses			Heterozygous for loci	Number of tetrads isolated from different perithecia (P)
(a) M 808–15	(a, c, spo, w, nap) × 6–36	(a^+, c^+, spo^+, w^+, nap^+)	a, c, spo, w, nap	29 from 10 P
(b) F_1	(a, c, spo, w, nap) × F_1	(a^+, c^+, spo^+, w^+, nap^+)	a, c, spo, w, nap	10 from 3 P
(c) F_1	(a, c, spo, w^+, nap) × F_1	(a^+, c^+, spo^+, w, nap)	a, c, spo, w	6 from 3 P
(d) F_1	(a, c, spo, w, nap) × F_1	(a^+, c^+, spo^+, w, nap)	a, c, spo,	3 from 3 P
(e) F_1	(a, c^+, spo^+, w, nap^+) × F_1	(a^+, c^+, spo^+, w^+, nap)	a, w, nap	6 from 2 P
(f) F_1	(a, c^+, spo^+, w, nap) × 6–36	(a^+, c^+, spo^+, w, nap^+)	a, nap	8 from 2 P

F_1 = ascospore strains of cross (a)

TABLE 3. *Results from the two-factor crosses in Table 2*

Combination of loci	Number of tetrads				
	PD	NPD	TT	Total	T%
a and c	14	6[a]	16	36	44·4
a and spo	13	10[a]	15	38	39·5
a and w	9	3[a]	17	29	58·6
c and spo	28	0	5	33	15·2
c and w	7	4[a]	17	28	60·7
spo and w	6	4[a]	15	25	60·0
nap and a	10	5[a]	34	49	69·4
nap and c	6	1	18	25	72·0
nap and spo	6	2	19	27	70·4
nap and w	5	1	18	24	72·0

PD = parental ditype tetrad. NPD = non-parental ditype tetrad. TT = tetratype tetrad. T% = number of TT × 100%/total number of tetrads (relative tetratype frequency).

[a] Number of NPD equals that of PD with 5% significance in the X^2-test.

sexual type, growth type and perithecial colour) could be analysed in combination with each other and with the character for naphthazarin production. The results, summarized in Table 3, are the following:

Mating type (locus a). More than 60 tetrads were analysed for mating type. The ratio obtained was always $4a^+:4a$. Two-factor analysis of the combinations locus a with locus c (sexual type), locus spo (growth type) or with locus w (perithecial colour) respectively gave no significant excess of the numbers of parental ditype tetrads over the number of non-parental ditype tetrads. There is no linkage of locus a with any of these markers. The post-reduction frequency (*PRF*) was found to be 0·397.

Sexual type (locus c). More than 40 tetrads were analysed for sexual type. The ratio obtained was always $4c^+:4c$. Two-factor analysis of the combination locus c with locus spo showed just 33 parental ditype tetrads. The lack of non-parental ditype tetrads indicates linkage of these two loci. The *PRF* was found to be 0·335.

Growth type (locus spo). More than 40 tetrads were analysed for growth type. The ratio obtained was always $4spo^+:4spo$. The linkage with locus c was mentioned above. The *PRF* was found to be 0·280.

Naphthazarin production. More than 50 tetrads were analysed for naphthazarin production. Twenty nine of these tetrads were isolated from the cross M 808–15 × 6–36, 16 tetrads from two different crosses between F_1-ascospore isolates of the cross M 808–15 ×

6–36, and 8 tetrads from a backcross of an F_1-isolate with 6–36 (Table 2). These 4 crosses were examined by two-factor analysis of the combination of capacity to produce naphthazarins with the other loci such as compatibility, sexual type, growth type and perithecial colour respectively. In all tetrads the ratio obtained for the capacity to produce naphthazarins or not was always $4nap^+ : 4nap$.

Parental ditype, non-parental ditype and tetratype tetrads could be found in each two-factor combination of capacity to produce naphthazarins with loci a, c, spo and w respectively (Table 3). Ascospore isolates of 10 tetrads derived from the cross 6–36 × 6–94 all produced naphthazarins in the same amounts as both parent strains. All ascospore isolates of two crosses between two non-naphthazarin-producing F_1-isolates of the cross M 808–15 × 6–36 (Table 2) were unable to produce naphthazarins. The culture filtrates of all naphthazarin-producing ascospore strains contained more than 3·0 μg total weight of naphthazarins per ml, but in culture filtrates of non-naphthazarin-producing ascospore strains no naphthazarin could be demonstrated (lowest determinable content 0·1 μg ml^{-1}).

The naphthazarin-producing ascospore strains of 6 tetrads of the cross M 808–15 × 6–36 were chosen for the quantitative analysis of the amounts of naphthazarin derivatives produced in 5 d old cultures. In each tetrad, the ascospore strains which produced naphthazarins, produced all the derivatives of the parent strain 6–36 in comparable concentrations, derivatives in % total naphthazarins: fusarubin 53–78%, novarubin 8–34%, norjavanicin and javanicin 2–12% each).

Degree of pathogenicity

The ascospore isolates of the 50 tetrads analysed for naphthazarin production were also examined for their degree of pathogenicity. The isolates of the cross M 808–15 × 6–36 showed degrees of pathogenicity corresponding not only to those of the parent strains, but also to classes in between (Fig. 1A). In 13 tetrads, the degrees of pathogenicity of all eight ascospore strains were intermediate and did not differ from each other (5% significance in analysis of variance). In 16 tetrads, significant differences between the degrees of pathogenicity of the ascospore strains were recognized. The ascospore strains of these tetrads could be separated into two groups, one highly pathogenic, the other weakly pathogenic. The ratio between the numbers of strains in these two groups was different for the various tetrads: in only 6 tetrads the ratio was found to be 4 : 4, the ratios of the other tetrads varied between 2 : 6 and 6 : 2. Crosses between F_1-ascospore strains (Table 2, letters b and e) yielded progenies, whose degrees of pathogenicity were distributed in classes beyond those of the intermediate parents (Fig. 1B). A backcross of an F_1-isolate with 6–36 (Table 2, letter f) yielded progenies, whose degrees of pathogenicity were distributed in classes between those of the parents (Fig. 1C).

Relationship between inheritance of naphthazarin production and pathogenicity

Only in 6 tetrads of the cross M 808–15 × 6–36 was naphthazarin production correlated with pathogenicity (Table 4). The naphthazarin-producing ascospore isolates from each of these tetrads showed significantly higher degrees of pathogenicity than those of non-naphthazarin-producing ascospore strains. All other tetrads showed no correlation between naphthazarin production and pathogenicity. The ratio between the number of naphthazarin-producing and non-naphthazarin-producing ascospore strains was approximately 50 : 50 in all pathogenicity classes (Fig. 2). A slight trend for classes of high pathogenicity to contain more naphthazarin-producing ascospore strains was noted, yet the proportion of naphthazarin-producing ascospore strains is never significantly different from 50% (5% significance in X^2-test).

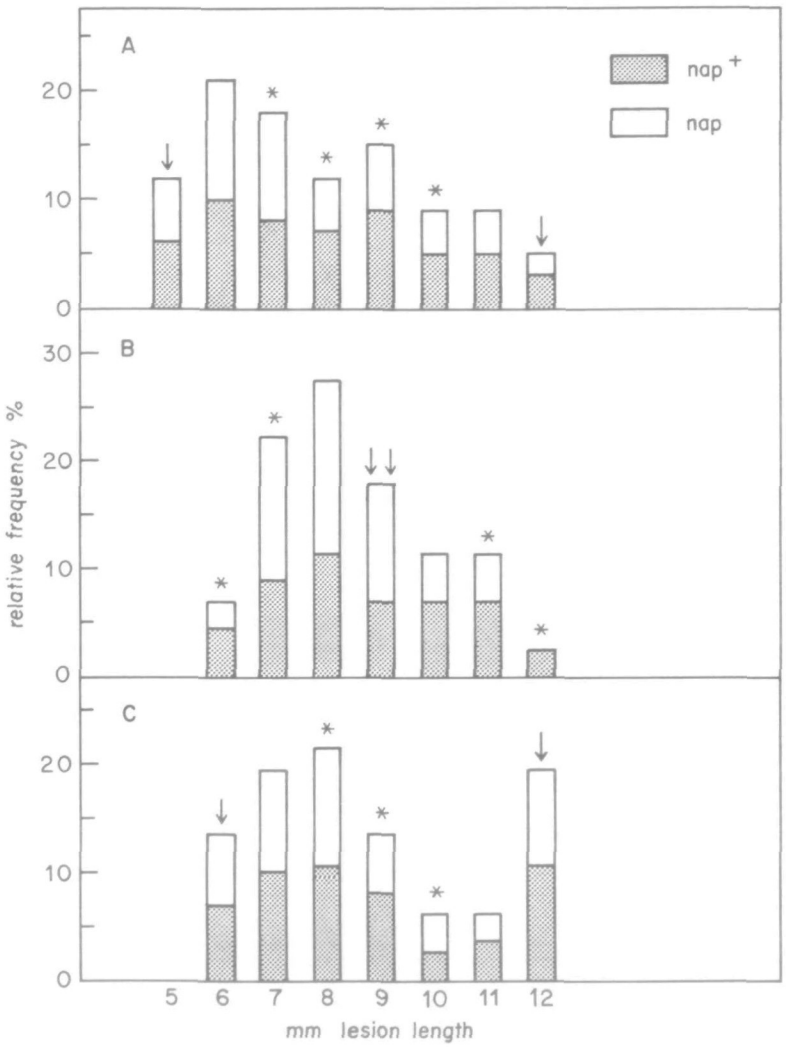

FIG. 1. Distribution of the ascospore isolates from different crosses with relation to their degree of pathogenicity (lesion length). A: cross M 808–15 × 6–36. B: cross between middle pathogenic F_1 ascospore isolates (Table 2, letter b). C: backcrossing of a low pathogenic F_1 ascospore isolate with 6–36 (Table 2, leffer f). Abscisse: classes with the intervals $(x - 0{\cdot}5/x + 0{\cdot}5)$. * Ascospore isolates in this class differ from their parents with 5% significance in the t test. † Degree of pathogenicity of the ascospore used as parent. nap$^+$: capacity to produce naphthazarins. nap: loss of this capacity.

DISCUSSION

The results of genetic analysis of the loci a, c, spo, w agree quite well with those reported by other authors. Georgopoulos (1963) showed, that the locus a (mating type) is not linked with locus c (sexual type), using additional markers such as resistance to PCNB. His assumption, that locus w (perithecial colour) was situated on a third linkage group, is confirmed by our results. Also the findings of Hansen and Snyder (1943), stating a linkage relationship of locus spo (growth type) with locus c, are confirmed by our investigations. Post-reduction frequencies were in good accordance with the data of Georgopoulos (1963), except that for locus c, which differed markedly. However, one cannot presume that there are two different

TABLE 4. *Degree of pathogenicity and naphthazarin production of the ascospore strains of six tetrads in which these characters are correlated*

Naphthazarin production	Degree of pathogenicity[a] of the ascospore strains					
	Ascus 06·3	06·6	06·16	06·24	06·26	06·28
+	10·2 ± 2·9	8·9 ± 1·9	9·4 ± 0·8	10·4 ± 2·7	11·3 ± 3·4	12·0 ± 2·3
+	12·3 ± 3·4	8·3 ± 2·9	8·2 ± 2·1	10·2 ± 2·5	11·2 ± 1·2	12·5 ± 2·0
+	10·3 ± 3·1	10·5 ± 2·7	7·8 ± 1·6	11·3 ± 2·3	8·1 ± 2·9	9·5 ± 2·5
+	9·6 ± 2·3	9·7 ± 2·5	7·2 ± 0·8	11·7 ± 3·1	8·8 ± 2·1	10·1 ± 2·3
−	6·0 ± 1·4	6·6 ± 1·5	6·6 ± 1·9	6·0 ± 1·0	6·7 ± 1·0	6·7 ± 1·3
−	6·6 ± 1·7	6·5 ± 1·5	5·4 ± 0·7	6·7 ± 0·9	6·3 ± 1·2	5·2 ± 0·7
−	5·9 ± 1·0	6·4 ± 0·7	5·2 ± 0·7	6·1 ± 0·8	5·4 ± 0·7	5·0 ± 0·5
−	6·6 ± 1·3	6·5 ± 1·4	5·0 ± 0·5	6·4 ± 1·5	5·8 ± 0·7	5·0 ± 0·5

[a] Average and variation of the lesion length in the stem infection of 20 plants per ascospore strain (data from two essays).

The degrees of pathogenicity of the naphthazarin-producing ascospore strains were significantly different from those of the non-naphthazarin-producing ones, in all 6 asci (5% significance in the analysis of variance).

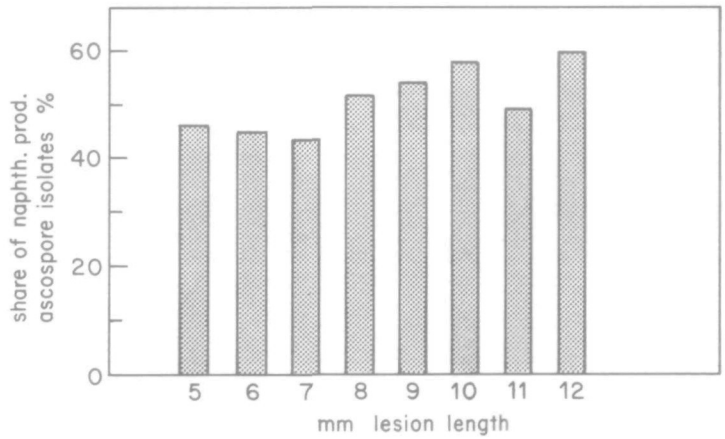

FIG. 2. Proportion of naphthazarin-producing ascospore isolates in the pathogenicity classes (see explanations Fig. 1). The share of naphthazarin-producing ascospore isolates is 50% in all classes with 5% significance in the X^2-test.

loci for unisexual femaleness. The cause of this difference is more likely to be the relatively small sample size or localization of chiasmata.

In all the tetrads analysed for naphthazarin production a 4:4 ratio for naphthazarin-producing to non-naphthazarin-producing ascospore strains was found. Two-factor analysis of naphthazarin production with all other markers (mating type, sexual type, growth type, perithecial colour) always gave parental ditype, non-parental ditype and tetratype tetrads. Thus, loss of capacity of M 808–15 to produce the naphthazarins, fusarubin, novarubin, javanicin, norjavanicin, is caused by mutation of a single locus (locus nap). Tetratype tetrad frequencies indicated no close linkage of the locus nap with any other loci considered in this paper. The locus nap appears to be somewhat distant from its centromere.

As strain M 808–15 has lost not only the production of fusarubin, novarubin, norjavanicin and javanicin but also of isomarticin and marticin (the two other toxins produced by the

wild-type strain *F. solani* var. *martii*) it is assumed that these two substances are also controlled by the locus nap. It also seems likely that this mutation inhibits an early step in the biosynthesis of a common precursor, most probably a polyketide, which could be transformed into different naphthazarin products by oxidation, addition or degradation of chemical side groups (Kurobane, Vining, McInnes, and Smith, 1978; Kern and Naef-Roth, 1965; Stoessl, 1981).

The results of the genetic analysis of the degree of pathogenicity of the progeny of *N. haematococca* indicates that this character can be regarded as a biometric or quantitative character because it was not expressed in two discrete states but varied continuously. Such characters are often subject to oligogenic or polygenic controls (Mather and Jinks, 1971). In the literature, references to investigations of the inheritance of the degree of the pathogenicity for the same variety are rather scarce. Only Nelson and Kline (1969) indicate that genes for host specificity in *Cochliobolus heterostrophus* can show markedly different degrees of pathogenicity in the progeny.

The relationship between inheritance of toxin production and of pathogenicity for the same host variety are not reported in the literature. On the other hand, Scheffer, Nelson, and Ullstrup (1967) showed by analysis of interspecific hybrids that host specificity in *Cochliobolus victoriae* and in *C. carbonum* was correlated with the ability to produce host-specific toxins. Our results of the tetrad analysis indicate that capacity to produce the weakly phytotoxic compounds novarubin, fusarubin, norjavanicin and javanicin is not a major factor in determination of the degrees of pathogenicity. This confirms the results of physiological experiments of Roos (1976). Kern (1976) suggested a more important role for the highly phytotoxic naphthazarin compound isomarticin in relation to pathogenicity. As crosses with the isomarticin-producing wild-type strain of *Fusarium solani* var. *martii* did not yield fertile perithecia, the influence of this compound could not be studied genetically. Strains 6–36 and 6–94 of *Nectria haematococca* are as pathogenic as the wild type strain M 808–1 of *Fusarium solani* var. *martii*, which additionally produces isomarticin and marticin (Holenstein, 1982). If isomarticin really is an essential factor for a high degree of pathogenicity, then strains 6–36 and 6–94 must possess several additional factors which give all together an equivalent degree of pathogenicity. This is quite possible because of the different origins of these strains.

ACKNOWLEDGEMENTS

We thank Prof. H. Kern and F. Würgler (ETH) and Dr. I. Smith (EPPO) for help in preparing this manuscript. This work was supported by a grant from Swiss Federal School of Technology (0.330.079.06/2).

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